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The Use of RNA Interference to Mitigate Pulmonary Fibrosis in Response to Asbestos Exposure

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THE USE OF RNA INTERFERENCE TO MITIGATE PULMONARY FIBROSIS IN RESPONSE TO ASBESTOS EXPOSURE

By

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ABSTRACT

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Faculty Mentor: Dr. Elizabeth A. Putnam

The adverse health effects of exposure to asbestos are widely known and have been well documented. When a person is diagnosed with asbestosis, a chronic lung disease caused by inhaling asbestos fibers, few treatment options exist, none of which halt or reverse the progression of the disease. The rapidly growing field of gene therapy offers new avenues for potential treatments worthy of investigation. The detrimental effects of asbestos exposure are due to the physiological response of the lungs to asbestos fibers in the form of fibrosis, a result of excess extracellular collagen deposition. A protein called SPARC (Secreted Protein Acidic and Rich in Cysteine) has been identified in previous studies as being a matricellular protein involved in the fibrotic response following asbestos exposure. Using the principle of RNA interference, we aimed to knock down the expression of SPARC in our studies, hypothesizing that a reduction in SPARC expression would yield a reduction in fibrosis. In our *in vitro* experiments, we prepared several viral constructs containing a SPARC-specific short hairpin RNA (shRNA), and identified the most effective construct using primary mouse lung fibroblasts. The most effective construct was used to infect asbestos-exposed and control mice. The presence of fibrosis was measured in three distinct ways:

1. Histologically to visually observe the presence of fibrosis,
2. Using RT-PCR to measure the presence of SPARC mRNA,
3. Using a Western Blot to measure the presence of collagen.

We expected to observe that the mice treated with the SPARC shRNA containing virus experienced less fibrosis, and had less SPARC mRNA and collagen present in their lungs than control mice. Unfortunately, none of the mice in our study developed fibrosis after asbestos exposure, as had been previously demonstrated. We intend to reevaluate our instillation method, among other procedures, before proceeding with future studies.

Introduction

Asbestos is a broad term used to describe a group of silicate minerals in the form of thin fibers (1). These minerals are naturally occurring and were mined extensively in the early 1900's for their substantial commercial utility due their high tensile strength and heat resistant properties (1) (2). Asbestosis can be defined as the interstitial fibrosis caused by the inhalation and deposition of asbestos fibers in the lungs (1). The most common symptom of this disease is dyspnea, though a nonproductive cough and chest pain are also typical (1).

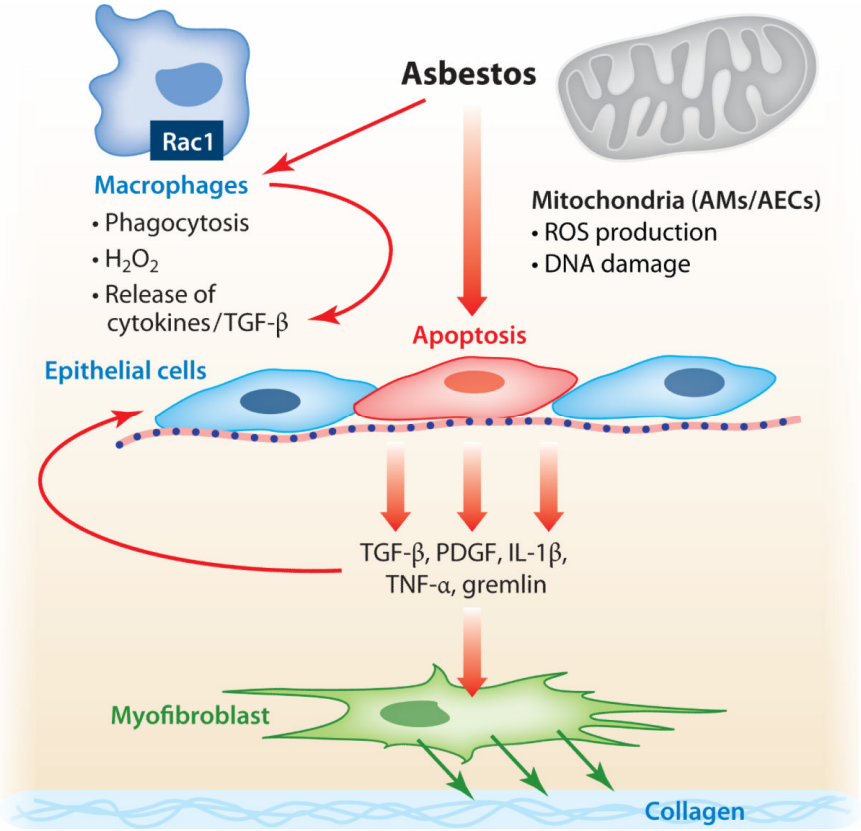
The molecular mechanisms underlying asbestosis are complex and have yet to be fully elucidated. To date, we understand that asbestos triggers the accumulation of alveolar macrophages and an inflammatory response (3). Subsequently, broad pulmonary involvement is observed, indicated by the loss of alveolar epithelial cells, fibroblast proliferation and collagen deposition (See Fig. 1) (3). Due to the long, thin shape of the asbestos fibers, the alveolar macrophages are unable to ingest them. This frustrated phagocytosis results in the release of transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), among other growth factors and cytokines; these encourage the deposition of collagen in the walls of the alveoli and elsewhere, resulting in fibrosis (3). In addition to the release of macrophage products, recent studies demonstrate that alveolar epithelial cell apoptosis is sufficient to induce pulmonary fibrosis (3).

One protein that appears to be a promising target in the fibrosis pathway is SPARC (Secreted Protein Acidic and Rich in Cysteine), also known as osteonectin or BM-40. This 43kD matricellular protein is known to interact with cell-surface receptors and the extracellular matrix (ECM) without contributing to ECM structure and is activated during tissue renewal, remodeling and repair as well as embryonic development (4). In her doctoral thesis of 2009 entitled, *The Influence of SPARC on Collagen Deposition in Asbestos-Induced Pulmonary Fibrosis*, Smartt summarizes the current understanding of this protein as follows, "Since its discovery, SPARC has been shown to function as a modulator of growth factor activity (5) (6) (7) (8), a counter-adhesive protein (7) (9), a modulator of cell proliferation (10) (6) and migration (11) (12) a regulator of the extracellular matrix (13) (14), and a cell cycle inhibitor (10)" (4). Additionally, SPARC has been shown to stimulate the TGF-β signaling system (8).

A 2010 study used RNA interference to knock down the expression of SPARC in a murine model with bleomycin-induced fibrosis and demonstrated that inhibition of SPARC

significantly reduced pulmonary and epithelial fibrosis and collagen expression (15). These results were promising, but would they hold true in an asbestos-induced fibrosis model? However, a study published in 2011 indicated that the effect of SPARC might not be as clear as was previously thought. Those results established that SPARC-inhibited mice demonstrate decreased collagen deposition compared to controls at low doses of bleomycin, but increased collagen deposition at high doses (16). A 2013 study showed that, in addition to SPARC stimulating TGF-β as was demonstrated in 2003, SPARC expression is also upregulated by TGF-β (17). The study also found that SPARC expression was necessary for the production of hydrogen peroxide (H_2O_2) in fibroblasts, which contributes to epithelial damage (17). Clearly, these findings collectively demonstrate that SPARC plays a role in the development of fibrosis.

The aim of this study was to determine the effect of SPARC inhibition on collagen deposition and pulmonary fibrosis in a murine model after asbestos-induced fibrosis. Crocidolite asbestos was used for these studies to allow for comparison with other data in the field.



(3)

Figure 1: A Hypothetical Model of Asbestos-Induced Pulmonary Fibrosis (3)

Methods:

Amphibole: Crocidolite asbestos was obtained from the Research Triangle Institute (Research Triangle Park, NC). The fibers were 0.16μm in diameter and 4.59μm in length with an aspect ratio of 34.05. Samples were prepared in phosphate-buffered saline (PBS) with a pH of 7.4 and sonicated before instillation (4).

Mouse Treatment: All animal protocols were approved by the Institutional Animal Care and Use Committee. C57BL/6 mice at 8 to 10 weeks of age were divided into two groups and exposed via inhalation to either 100μg of crocidolite asbestos in 30μL of sterile PBS or PBS alone as a control. After two months, the mice were exposed via inhalation to one of three treatment options: 1. Virus containing a SPARC-specific shRNA construct, 2. Virus containing an shRNA with no known mammalian gene targets as a negative control, 3. PBS as a control. The mice were euthanized one month after receiving the virus treatment and their lungs harvested for study. The right lung was saved for histology. The left lung was divided and the superior lobe was snap frozen in liquid nitrogen for RNA isolation while the inferior lobe was placed in PBS-TDS for later protein analysis. Twelve mice began this procedure as group 1 on the 25th of November, 2014. A second group followed on the 3rd of December, 2014.

Lentivirus Production: A plasmid containing a SPARC-specific shRNA construct was purchased from Sigma Aldrich and amplified in *E.coli*. The resulting plasmid DNA was isolated and transfected into 293 FT cells along with the viral backbone. Selection with pen/strep insured only the cells infected with virus survived. The virus was subsequently harvested and stored at -80˚C. The virus was titered using A549 cells with pen/strep selection and the colonies remaining after 1 week were stained with crystal violet and counted.

Histology: The lungs were not perfused prior to embedding in paraffin. Sections of 7μm were taken about 35μm apart, placed on slides and stained with trichrome stain to visualize collagen. The sections were examined under light microscopy.

RNA Isolation and Quantitative RT-PCR: Lung tissue was homogenized in 1mL of Trizol, isolated following the manufacturer's protocol (Invitrogen, Carlsbad CA), purified using the RNeasy kit (Qiagen, Valencia CA) and subsequently treated with DNAse (Qiagen). For qRT-PCR, initially first strand synthesis of cDNA was accomplished using a First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). Target cDNA was subsequently amplified using predesigned TaqMan probes (ABI, Foster City, CA) for Collagen3A1, SPARC and β-Actin as a transcript control. qPCR was performed on an iQ5 Optical System (Bio-Rad, Hercules CA) (4).

Protein Isolation and Western Blotting: Lung tissue was homogenized in lysis buffer PBS-TDS (PBS, Triton X-100, sodium desoxycholate, sodium dodecyl sulfate, EDTA, phenylmethylsulphonylfluoride and protease inhibitor) and centrifuged to isolate protein. Total protein concentration was measured using a Bio-Rad assay. Lung samples were separated by electrophoresis through 4-12% Bis-Tris NuPAGE gels (Invitrogen, Carlsbad CA), transferred to PVDF membrane (Milipore, Billerica MA), and blocked in 5% milk PBS-Tween20. An antibody specific to collagen was used to detect protein levels (4).

Statistical Analysis: Data were analyzed using a two-way ANOVA with a Bonferroni adjustment.

Results

Quantitative RT-PCR to determine the mRNA expression of SPARC was performed in order to conclude whether the shRNA virus was effective at reducing SPARC expression. Additionally, quantitative RT-PCR was used to measure the expression of collagen in the various treatment groups. Initial results did not follow any expected pattern, but upon statistical analysis using a two-way ANOVA with a Bonferroni adjustment, it was determined that there was no difference in SPARC expression between the treatment groups. We would have certainly expected to see a difference in SPARC expression between the asbestos-treated and control mice that received neither virus, as previous results in our laboratory have shown that asbestos induces increased SPARC expression and fibrosis. However, there are no statistically significant differences in SPARC expression among any of the six treatment groups.

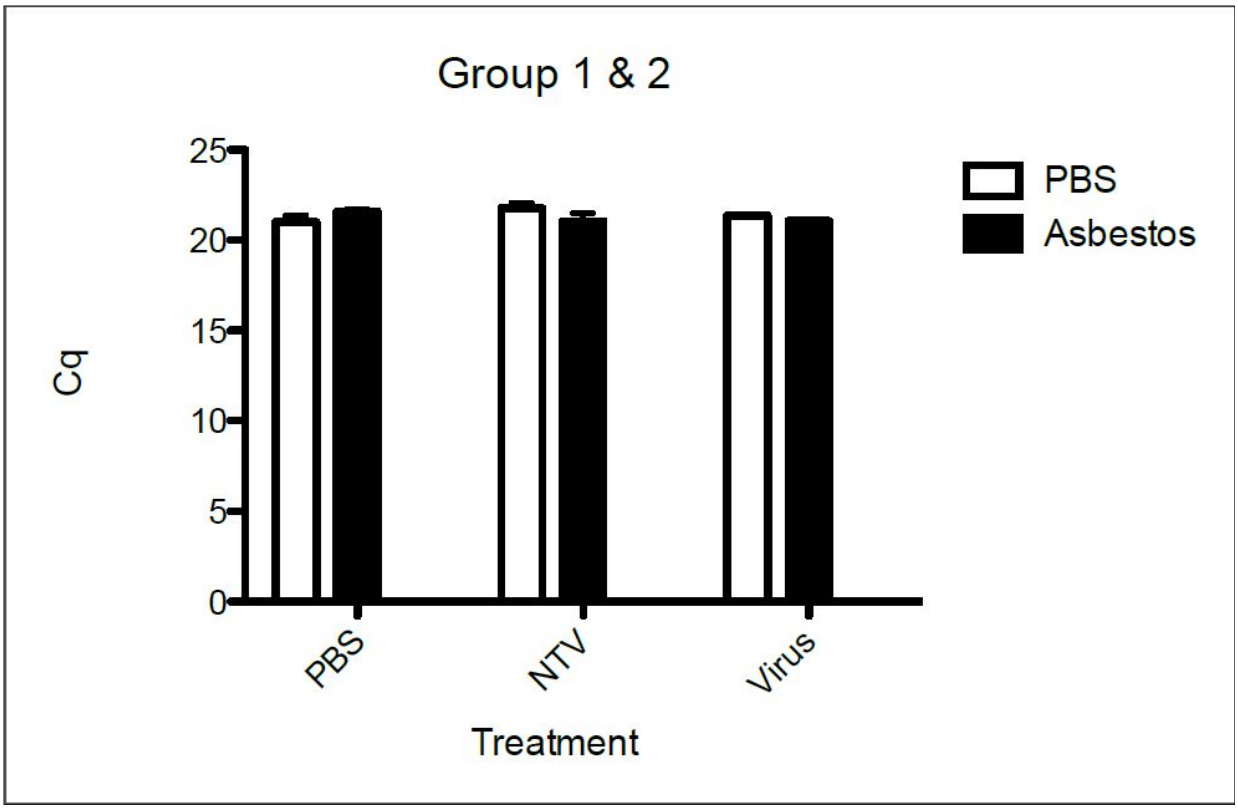


Figure 2: Graph of a 2-way ANOVA test combining groups 1 and 2. SPARC expression is measured on the y-axis by Cq, quantification cycle. Black bars indicate mice receiving asbestos treatment and white bars indicate mice that received PBS as a control in the first stage of the experiment. The x-axis categories indicate the treatments in the second stage of the experiment, "PBS" being the control again, "NTV" being the negative control virus and "Virus" being the virus containing the shRNA for SPARC inhibition.

Examination of histology slides stained with trichrome stain to view the presence of collagen was performed to visually observe the effect of asbestos and SPARC-inhibition on inflammation. Blue coloration indicates the presence of collagen. Similar to the quantitative RT-PCR results, examination revealed no difference among the treatment groups.

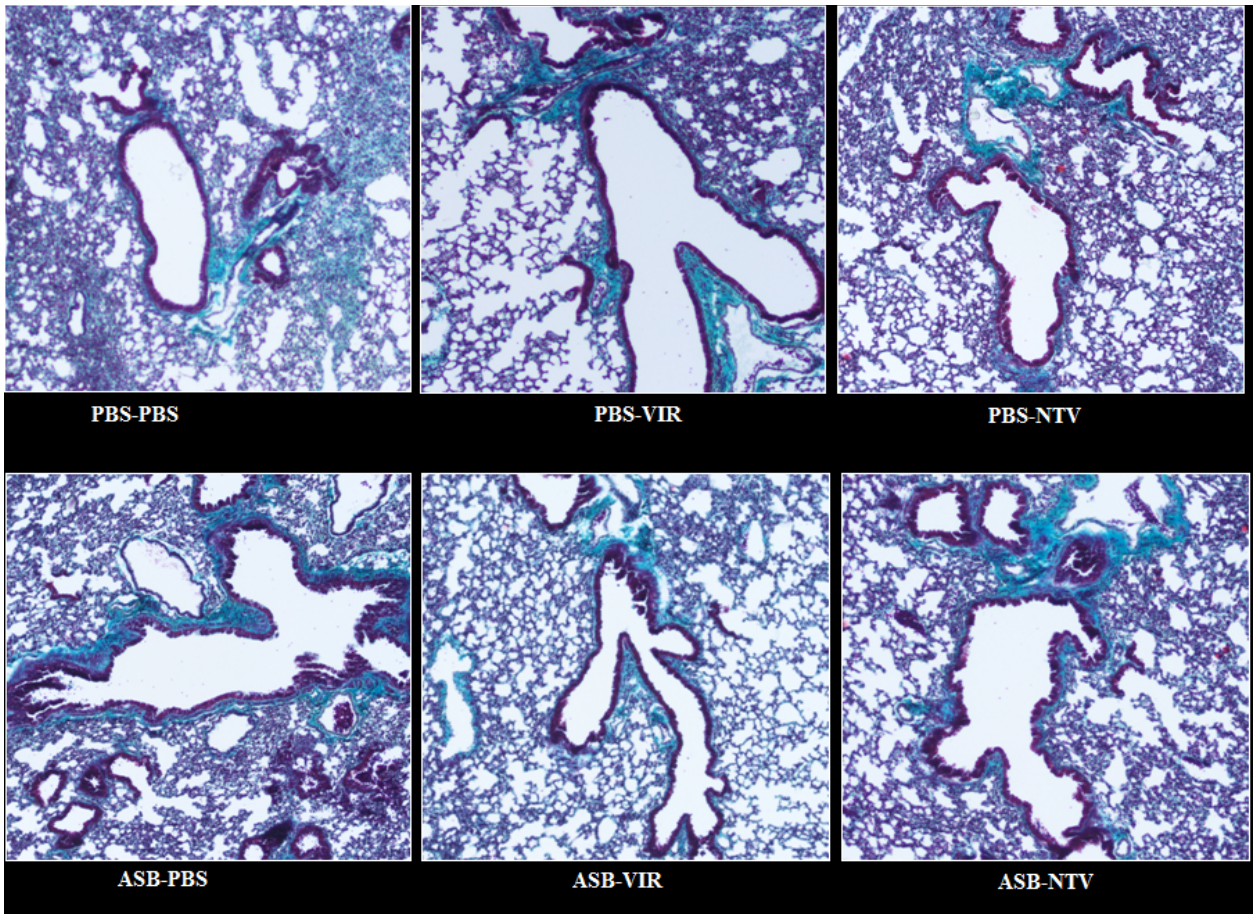


Figure 3: Histology slides of each treatment group, comparing similarly sized airways. Fibrosis is indicated by the presence of collagen (stained blue) and increased cellularity. Note that the lungs were not perfused prior to embedding in paraffin, thus cellularity may appear denser than is typically seen in the literature. However, comparing the presence of collagen and cellular density between treatment groups, there do not appear to be noticeable differences.

A Western Blot was also performed in order to quantify the presence of the collagen protein among treatment groups. However, despite repeated attempts the blot was too smeared to use accurately for protein quantitation. This may be due to protein degradation or another unknown cause. Regardless, based on histological and quantitative RT-PCR data, it is unlikely that differences in collagen among treatment groups would have been found to be statistically significant.

Discussion

The lack of any observable differences in SPARC expression or collagen deposition among the treatment groups indicates that fibrosis did not occur in any of the mice. Previous

results in this lab and a plethora of studies in the literature have consistently demonstrated the development of fibrosis after asbestos exposure, thus it is likely that our methodology is off, rather than our conceptual approach. Previous experiments in this lab used intratracheal instillation as the method of asbestos exposure, which required a surgical incision into the trachea of the mice in order to instill the asbestos (4). For this experiment, we used a new method of asbestos instillation to minimize harm to the mice. This inhalation method did not involve any surgery, instead the asbestos solution or control was placed on the back of the throat of an anesthetized mouse, causing the mouse to inhale it into their lungs. However this method may not be as reliable or efficient as the previous method. Perhaps the solution ended up in the esophagus instead, or was more easily coughed back up by the mice. It seems that others who use the inhalation method with success do so with repeated exposures over a period of time. It is possible that this method is simply less efficient and, thus, multiple exposures or higher dosages are needed to see the effect that was noted with the previous intratracheal method. Regardless, our lab will need to evaluate our instillation paradigm and demonstrate its efficacy before moving forward with more experiments.

In addition to reattempting this experiment with revised methodology, we would also like to begin investigating other players in the fibrosis pathway, such as Smad-2 or Reactive Oxygen Species (ROS) to better elucidate the mechanism of fibrosis in response to asbestos. We would also like to begin *in vitro* experiments using human-tropic viruses and human cell lines, to investigate the possibility for mitigation of the fibrosis response and treatment of asbestosis in humans.

Conclusion

Despite our lack of ability to draw conclusions from this experiment due to an ineffective method of inducing fibrosis, it is clear from the literature that SPARC provides a promising target for inhibition, in the reduction of fibrosis. Further investigation by our lab and others will be necessary for the advancement of this valuable work.

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